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Genetic diversity and population structure of Marsh Grassbird (*Locustella pryeri sinensis*) in China

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Abstract: We used sequences of mitochondrial control region (807bp) in 75 samples from three breeding colonies and one wintering population to investigate the genetic diversity and population structure of Marsh Grassbird (*Locustella pryeri sinensis*) in different regions of China. Marsh Grassbird retained a moderate amount of haplotype (0.759 ± 0.056) and nucleotide diversity (0.002). The results of F_{ST} among 3 phylogeographic units and Φ_{ST} between breeding and wintering sites revealed little evidence of genetic distinction between different colonies. Neither UPGMA tree structure analysis nor Network picture analysis showed obvious divergence between populations at different locations. Analysis of molecular variance also showed a lack of regional subdivision within *Locustella pryeri sinesis*, 98.5% of source of variation within populations and only 1.5% among populations. The neutrality test showed negative Fu's FS value, which, in combination with detection of the mismatch distribution, suggested that population expansion occurred in the evolutionary history of this species. This hypothesis was further supported by Tajima's D test and Fu's test (D = -1.80, p = 0.02; Fs = -22.11, p = 0.001), this expansion was estimated to occur about 28,700 years ago.

Key words: Marsh Grassbird; *Locustella pryeri sinensis*; genetic diversity; population structure; mitochondrial DNA (mtDNA); gene flow

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Introduction

The population genetic structure of a species depends on both historical events and current processes (Avise 1994; Templeton et al. 1995). A good understanding of population genetic structure can support design of an effective species conservation program. Molecular markers provide important measures of population structure and geographic differentiation and have been employed to assess questions in evolutionary and conservation biology (Avise 1994; Avise and Hamrick 1996). Mitochondrial DNA (mtDNA) is an effective molecular marker for evolutionary studies at recent levels of divergence (Avise 1994). Although some authors (e.g. Edwards et al. 2005) are calling for multi-locus assessments of phylogeographic structure, the best locus for revealing phylogeographic pattern is mtDNA because of its rapid coalescence (Zink et al. 2008, Barrowclough *et al.*, 2005). Thus it remains important to document phylogeographic structure with mtDNA gene trees (Zink et al. 2008).

The Marsh Grassbird (*Locustella pryeri*) breeds in marshes with reed and sedge beds in the Far East (Fujita et al. 1997; Li and Wang 2007). Population sizes are typically small and the species is considered to be declining as a result of wetland destruction at breeding and wintering grounds. Grass Marshbird is listed as globally near threatened (IUCN 2010). To develop adequate conservation strategies for felid species, it is essential to understand its genetic diversity and geographic patterns of population subdivision.

Because the control region may be the most rapidly evolving part of mtDNA, having a substitution rate ranging from 5 to 10 times higher than other parts of the mtDNA molecule (Aquadro et al. 1983; Wenink et al. 1996), it has been successfully used for examining intraspecific relationships and evolutionary relationships between closely related species (Avise 2000; Starkey et al. 2003).

In China, Marsh Grassbird has been recorded breeding in Liaoning and Heilongjiang Provinces, and wintering in Hubei Province (MacKinnon et al. 2000; Zheng 2005). Recent investi-

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gations suggested that Marsh Grassbird has three breeding populations in China: In addition to the previously identified breeding populations at Zhalong (Heilongjiang) and Shuangtai Hekou (Liaoning), Poyang Lake (Jiangxi Province) has been recognized as a new breeding ground after formerly being considered only a wintering site. Poyang Lake now supports the largest breeding population of Marsh Grassbird. A small population of Marsh Grassbird was recently found nesting on Chongming Island at the river mouth of Yangtze (Gan et al. 2006). After further study, this might also prove to be a separate breeding population. This study addresses the population genetic structure of Marsh Grassbird and its relation to geographic location.

Materials and methods

Study Area and Sampling Methods

We studied three breeding populations: Zhalong (46°52′–47°28′N, 123°47′–124°37′E), Shuangtai Hekou (40°45′–41°10′N, 121°30′–122°00′E), Nanjishan (28°52′–29°06′N, 116°10′–116°23′E) and one wintering population: Nanjishan (28°52′–29°06′N, 116°10′–116°23′E). Seventy-five samples were collected from three breeding and one wintering locality (Fig. 1). Most of the samples were feathers and blood, while some were muscle tissue from dead nestlings.
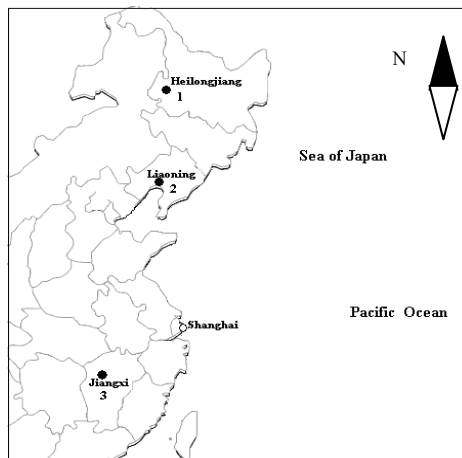

Fig. 1 Geographic locations of three sampling sites inChina
1: Zhalong National Nature Reserve (NNR) in Heilongjiang, 2: Shuangtai Hekou NNR in Liaoning, 3: Nanjishan NNR in Jiangxi

DNA Extraction, Amplification and Sequencing

DNA was extracted using a standard proteinase-K phenol/chloroform technique (Sambrook and Russel 2001). The partial sequence of mitochondrial control region in Marsh Grassbird was amplified by primers H1248 (5′-CATCTTCAGTGTCATGCT-3′) and L437 (5′-CTCACGAGAACCGAGC TAC-3′), which were designed for oscine passerines (Tarr 1995). Polymerase chain reaction (PCR) was performed in volumes of 50 μl which included 10–50

ng of total genomic DNA, 0.20 mM of each nucleotide, 3.0 mM MgCl$_2$, 0.6 M of each primer, 0.5 units of Taq DNA polymerase and 50 ng total DNA. The PCR was run under the following conditions: 30 s at 94°C, 45 s at 50°C, 60 s at 72°C (35 cycles). Before the cyclic reactions the samples were incubated at 94°C for 4 min, and after completion at 72°C for 7 min. PCR products were purified with PCR purification kits and ligated to pMD-18 T vectors (TaKaRa, Japan) according the manufacturer's instruction, and then amplified in E. coli Positive colonies before sequencing with an ABI 3700 automated sequencer. A sequence was accepted for further analysis only if it was identical in more than three colonies. All sequence data were edited (removing vector sequence) by using software DNAstar 7.1.0. Notations were given to each sequence before they were submitted to GenBank.

Statistical Analyses

Sequences were aligned using Clustal W in Mega4 (Tamura et al. 2007). Nucleotide diversity (π) and haplotype diversity (Hd) were calculated with DnaSP 5 (Librado et al. 2009) for each population. The degree analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed using Arlequin 3.11 (Excoffier et al. 2005) with significance tested by 1000 permutations. F values (F_{ST}) and Φ_{ST} were calculated using Jukes and Cantor's distance (Kumar et al. 1993) through an analysis of molecular variance (AMOVA) approach, and the calculated mean number of migrating females between populations per generation (Nm), both as implemented in ARLEQUIN. Global tests of differentiation among samples were preformed in ARLEQUIN using Markov chain analyses (10,000 steps). A hierarchical analysis was carried out among groups and populations by analysis of molecular variance (AMOVA). Tajima's D (Tajima 1989) and Fu's FS (Fu 1997) tests were performed in the software package ARLEQUIN 3.11. Then the validity of the estimated demographic model was tested by the distribution of a SSD test (the sum of squared differences) between the observed and an estimated mismatch distribution, which was obtained by a bootstrap approach.

Phylogenetic analyses of different haplotypes were performed in Mega4 (Tamura et al. 2007). UPGMA trees were constructed using the Jukes and Canter method. Branch support in UPGMA was assessed using 1000 bootstrap replicates. Gamma correction parameters were obtained through Modeltest 3.7 (Posada et al. 1998). The haplotypes network picture was built through software Network 4.501 using Median-joining method (Bandelt et al. 1999; Forster et al. 2001; Polzin et al. 2003).

Results

Sequence Variation and Haplotype Diversity

From 75 samples taken from three separate colonies we obtained 807 bp of mtDNA control region (GenBank No.: EU009402-EU009431; GU166299-GU166343). In these, we detected 34 variable sites (4.21%), including 28 transversions

(A↔G = 14, C↔T = 14) and 2 transitions noted at positions 139 and 379. In addition, 3 deletions and 1 insertion were also detected. The frequencies of four bases (A, T, G, C) were 31.61%, 26.85%, 29.13%, 12.41%, respectively. Nucleotide diversity (π) was 0.004 ± 0.003，while the average number of pairwise nucleotide differences (k) was 1.106.

Among 75 individuals of Marsh Grassbird, there were 29 haplotypes whose frequency values were between 1.33 and 48%, defined by 34 variable sites (Table 1). The most common haplotype was shared by 36 individuals (48% of all sampled birds) from 4 localities spread from NE to SE China, and from breeding to wintering populations. Three other common haplotypes were shared by nine and two individuals from two and four localities, respectively. Nucleotide diversity and haplotype diversity was lowest in Zhalong samples and highest in Shuangtai Hekou (Table 2). Haplotype diversity (Hd) value of all samples was 0.759, ranging from 0.678 to 0.952 for all populations. Haplotype diversity was negatively and significantly influenced by sample size ($p = 0.032$). Samples from the wintering grounds in Nanjishan, showed slightly lower levels of nucleotide diversity than Shuangtai Hekou, but higher than two other breeding sites, including the same sites as the wintering area which was thought to include birds from different breeding localities.

Table 1. Variable sites in the control region segment of Marsh Grassbird. Origin of samples indicated by Shuangtai Hekou, Zhalong, Nanjishan breeding and Nanjishan wintering

code	Haplotype (bases number) 111111 2222333344455 556666777 / 23469355889 1277236748905 891349124 / 110104667372 6412381944250 004387430	Shuangtai Hekou (n = 7)	Zhalong (n = 30)	Nanjishan breeding (n = 17)	Nanjishan wintering (n = 21)	Total
A	CATTGAGTAATG TAA-AATTCCCAGAAGCTAATA	2	17	8	9	36
B	t........... ...-...g.....	1				1
C	.g......... ...-.........			1		1
D	....--......... .....g...		1			1
E	...c....... ...-......... ......gc.	1				1
F	....a....... ...-.........	1	3	2	3	9
G	.....g...... ...a.........	1				1
H	......c..... ...-.........				1	1
I	.......c.... ...-.........			1		1
J	..c....c.... ...-.........			1	1	2
K	.......g... ...-.........		1	1		2
L	........g.. ...-.........				1	1
M	..........c. ...-.....t..a	1				1
N	...........a ...-..c......			1		1
O	.... c..-.........				1	1
P	.......g... .-.-.........		1			1
Q	....--.........		1			1
R	......c.... ..--.........				1	1
S	......c.... ..-g......... ...c....			1		1
T	....-.g.......				1	1
U	....-..c......		1			1
V	.......g... ...-....t....		1			1
W	......c.... ...-.....t..			1		1
X	....-......g.		1			1
Y	....-...... -.........		2			2
Z	....-...... .g.......				1	1
AA	......c.... ...-......... ..a......		1			1
AB	....-......... ...t.....				1	1
AC	....--......... ....g				1	1
Total		7	30	17	21	75

Table 2. Sample size (N), number of haplotypes (nH), haplotype diversity (Hd), nucleotide diversity (π), standard deviation of n (sd), Tajimas'D statistics (D) and Fu's FS value for four populations of Marsh Grassbird

population	N	nH	Hd	π (sd)	D'	Fs
Shuangtai Hekou	7	6	0.952	0.00354(0.00087)	-1.60974	-4.26577
Zhalong	30	11	0.678	0.00096(0.00025)	-1.87584	-29.28324
Nanjishan wintering	21	11	0.814	0.00149(0.00027)	-1.96150	-26.52601
Nanjishan breeding	17	9	0.787	0.00192(0.00044)	-1.75021	-28.36625

Genetic Divergence and Gene Flow

The ranges of the nucleotide divergence, pairwise F_{ST}, and gene flow (Nm) among the 4 populations were 0%–8.99%, 0.096–0.354, and 5.561–426.970, respectively (Table 3). Mean F_{ST} value was 0.015 ($p > 0.05$), while Φ_{ST} between breeding groups and wintering group was 0.01035 ($p > 0.05$). The number of migrants Nm among Zhalong, Nanjishan breeding and wintering population was above 10 individuals per generation, indicating a large amount of gene flow (Table 4). The smallest amount of gene flow appeared between Zhalong and Liaoning, 5.56 migrating individuals, and the F_{ST} value between them was the largest ($F_{ST} = 0.090$, $p < 0.05$).

Table 3 F_{ST} and p-value (below the diagonal) and gene flow (above the diagonal) between four populations of Marsh Grassbird.

	Zhalong	Shuangtai Hekou	Nanjishan wintering	Nanjishan breeding
Zhalong	-	5.56052	25.18688	426.96976
Shuangtai Hekou	0.08992 (0.02441)	-	12.32539	13.17464
Nanjishan wintering	0.01985 (0.14941)	0.04057 (0.08398)	-	INF
Nanjishan breeding	0.00117 (0.40723)	0.03795 (0.13184)	0(0.53809)	-

Phylogeographic Population Structure

AMOVA showed a lack of regional subdivision within *Locustella pryeri sinesis*, with 98.54 percent of source of variation arising within populations, only 1.46% of variations were among populations. Tajima's D and Fu's FS statistics were all negative in the four sampled Marsh Grassbird populations (Table 2). We identified a unimodal mismatch distribution for the Marsh Grassbird in which Tajima's D and Fu's FS were all significantly negative (D = -1.799, p = 0.016; Fs = -22.110, p = 0.001). Demographic parameters estimated by mismatch analyses corresponded to a null model of population range expansion ($\tau > 0$, $\theta_1 > \theta_0$) that could not be rejected (both p values of the sum of squared differences and Harpending's Raggedness index exceeded 0.05).

UPGMA analysis of the different haplotypes inferred a gene-tree with no clades, all the haplotypes from different locations mixed together (Fig. 2), the differentiation between haplotypes was small, and this result was also supported by network picture analyses (Fig. 3). From the network construction of haplotypes, we inferred that haplotype A was an original haplotype, with all other haplotypes derived from it directly or indirectly. The frequency of this haplotype was near 50%, and portions of haplotype A were always the largest in each population.
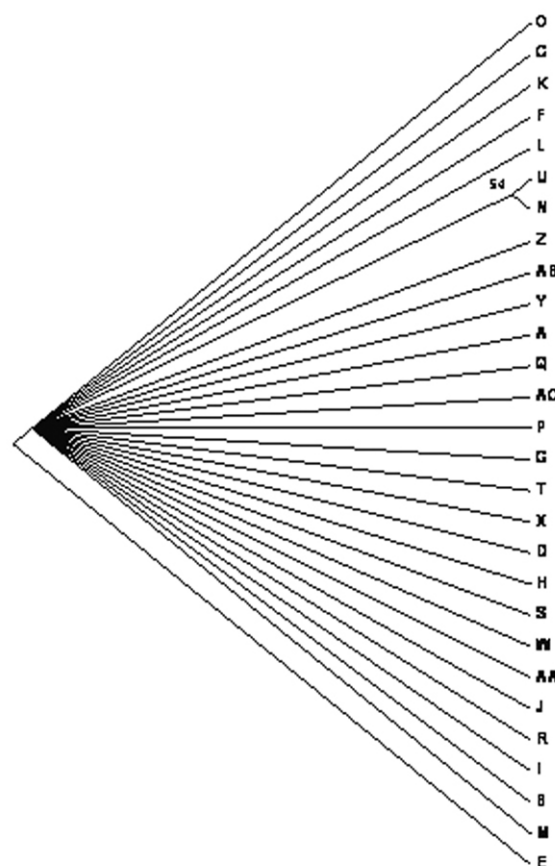

Fig. 2 Phylogenetic relationships among Marsh Grassbird mtDNA haplotypes constructed using UPGMA method based on 807 bases of the control region (cut off 50%). Haplotype codes as in Table 2
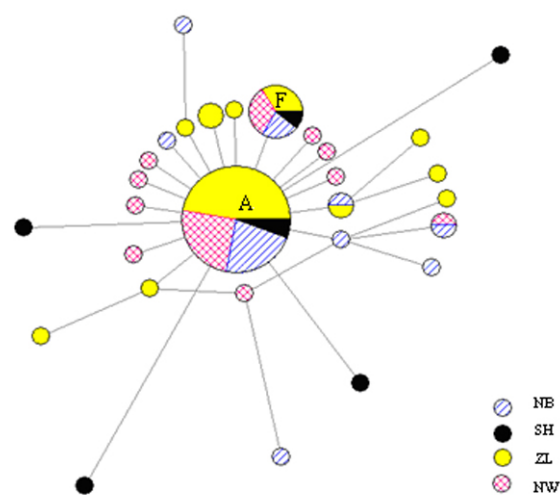

Fig. 3 Haplotypes network using the number of different mutations among 29 mtDNA haplotpyes of Marsh Grassbird Each circle represents a haplotype, circle sizes and colors are represented the number of samples and geographical distribution. Each line represents a mutational step.

Discussion

Though there were few base differences between individuals of Marsh Grassbird, the Marsh Grassbirds in China retained a moderate amount of haplotype (0.759 ± 0.056). Genetic diversity of Marsh Grassbird was high due to the expansion of the geographic distribution of the population, but the low nuclear diversity value and little haplotype differentiation suggested the history of population increase of Marsh Grassbird is recent. The results of genetic divergence analysis indicated the lack of differentiation between different populations, as well as between breeding and wintering populations. The smallest amount of gene flow and the largest F_{ST} value appeared between Zhalong and Liaoning, and this might be a result of the small sample size from the Liaoning population.

Lack of phylogeographic structure (Fig. 2, 3) and absence of quantitative population subdivision (Table 2) indicated high gene flow among populations of the Marsh Grassbird (Table 3). The demographic parameters estimated by mismatch analyses corresponded to a model of population range expansion. These suggest the population expansion of Marsh Grassbird, assuming an evolutionary rate of 14.8% per Myr for the control region (Wenink et al. 1996), occurred about 28 700 years ago.

The hypothesis of recent population expansion was supported by significantly negative Tajima's D and Fu's FS values. Star-like phylogenies, where the most common haplotype is also the most widespread, are usually interpreted as a signature of recent range expansion from a single source population. Nucleotide diversity appears to be higher in wintering than breeding populations, although there was one exception (Table 2). The mean value of F_{ST} between different sites was 0.015, and the genetic divergence between them was not significant (p = 0.108). This suggested that geographic spread did not result in genetic divergence. Gene flows were significant between the four sampled populations, indicating that separate breeding sites exchange individuals on their return from shared wintering grounds at Nanjishan, which is a possible source of expansion of the geographic distribution of Marsh Grassbird.

Because wintering populations probably comprise a mix of birds from different breeding populations, samples from Jiangxi wintering areas should contain more genetic diversity than samples from any breeding population. However, the Hd value of Shuangtai Hekou NNR of Liaoning Province was higher than that for the Nanjishan wintering population in Jiangxi Province. Hd values were significantly and negatively correlated with sample size (r = -0.963, p = 0.019). Detected differences between the genetic diversity values of the four populations and significant F_{ST} between Zhanglong (n=30) and Shuangtai Hekou (n=7) might have been caused by unbalanced sampling. Further knowledge of the genetic structures of populations at separate geographic locations is essential for understanding the historic population dynamics of this species，which would be helpful in the conservation management of this bird.

As predicted, most of the variation in control region sequences was found between individuals within populations. Hence, there was little evidence of mitochondria1 DNA differentiation between geographic locations. Poyang Lake of Jiangxi Province is the main wintering area for Marsh Grassbird in China, and the population is concentrated at Nanjishan NNR. However, there are probably other wintering locations for Marsh Grassbird in the lower Yangtze River basin in China (Collar 2001), more field investigations are needed for understanding how the current genetic structure forming of this bird in China.

Without regular monitoring a rare species may slip unnoticed into extinction (Grant et al. 2005). Marsh Grassbird is listed as globally near threatened by IUCN, but monitoring of the species population status has not been adequate at most distribution sites in China. The most serious threat to this species might be habitat loss and fragmentation, and human disturbance, which lead to the isolation of populations. The rapid rate of loss of marsh wetlands in each of the sampled provinces these years is clearly the most significant threat for this bird.

The discovery in China of a total breeding population of 5 000 pairs resulted in "down-listing" of the IUCN threat status from "Vulnerable" to "Near threaten" (IUCN 2010). However, the population sizes in two colonies (Zhalong and Shuangtai Hekou) are only about 100 and 60 breeding pairs (Li and Wang 2007), the recent expansion of population which supported by the genetic structure, indicates that some of the recently established populations would be small and therefore vulnerable to extinction. Therefore, protection efforts should focus on these sites, and the population in Jiangxi Province can serve as a source for birds to supplement populations at other suitable wetland habitats in China such as in Jilin Province which is located between Heilongjiang and Liaoning, where there is similar wetland habitat.

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